JOURNAL OF BACTERIOLOGY, Mar. 1978, p. 1237-1245
0021-9193/78/0133-1237$02.00/0

Vol. 133, No. 3
Printed in U.S.A.

Interspecies Transformation in *Bacillus*: Sequence Heterology as the Major Barrier

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Received for publication 11 October 1977

The relative contribution of DNA restriction and of sequence heterology as barriers to interspecies transfer of DNA was studied in the heterologous transformation of *Bacillus subtilis* recipients by DNA isolated from *B. globigii*. Transformants were obtained at very low frequencies in the evolutionarily nonconserved aromatic region; high cotransfer of linked markers was observed. New mutations were introduced into the *B. globigii* intergenote sequence in the resulting hybrids; these markers could be transformed with high efficiency by both *B. globigii* and *B. subtilis* DNA, representing a 10^5-fold increase in heterologous transforming efficiency. A restriction activity in *B. globigii* crude extracts inactivated the biological activity of *B. subtilis* and hybrid DNA but not *B. globigii* DNA in vitro, demonstrating different sites for restriction and modification between these species. In vivo, however, *B. globigii* and hybrid DNA transformed the *B. globigii* sequences in a hybrid recipient with the same efficiency. These results show that sequence heterology is the major barrier to interspecies transformation and that, in this system, enzymatic restriction does not prevent interspecies transformation.

Heterologous transformation, or the interspecies transfer of DNA, has been described in many systems. The distinguishing characteristic of these crosses is the low frequency of their occurrence compared with homologous transformation efficiencies. The relative efficiency of transformation (E.T.) of heterologous crosses has been used as a quantitative measure of taxonomic relationship (41). Even between the same species, however, the E.T. can vary by a factor of 10^3 or more depending on the marker selected; certain regions of the chromosome, including the ribosomal and tRNA genetic markers, are transformed heterologously at much higher frequencies than other markers. These regions show higher levels of homology between species as measured by DNA-DNA or DNA-RNA hybridization, as if these regions are evolutionarily conserved between species (9, 10, 12). Other nonconserved regions, including most auxotrophic markers, show low homology and demonstrate low heterologous transformation efficiencies (9).

Three major barriers to heterologous transformation have been proposed: (i) differential uptake of homologous and heterologous DNA; (ii) enzymatic restriction of heterologous DNA inside the cell; and (iii) DNA sequence nonhomology leading to reduced recognition and a chromosomal integration of heterologous DNA. The first factor, discrimination at uptake, does not occur for any systems showing measurable heterologous transformation (25, 28, 29), although discrimination against uptake of unrelated *Xenopus laevis* and *Escherichia coli* DNA has been reported in *Haemophilus influenzae* (33). Site-specific cleavage of foreign DNA in vitro by purified restriction endonucleases is well known (2, 26); however, site-specific cleavage of bacterial transforming DNA has not been demonstrated in vivo. In both *Haemophilus* (16, 38) and *Bacillus* (39), manipulation of the restriction and modification phenotypes does not significantly affect heterologous and homologous tranformation frequencies. Similarly, genetic experiments involving pneumococcus-streptococcus transformation (30) suggest that enzymatic restriction has only a slight role in lowering heterologous transformation frequencies. These results, together with the correlation between the extent of DNA-DNA hybridization and heterologous transformation efficiency (10, 12), argue strongly that sequence nonhomology is a major barrier to interspecies transformation (32).

We have studied heterologous transformation of evolutionarily nonconserved genetic markers in *Bacillus subtilis* Marburg strains by DNA from the related species *B. globigii*. These

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crosses were accomplished at exceedingly low frequencies, but, in contrast to crosses for evolutionarily conserved markers, with very high cotransfer of linked markers. Transformations were performed with the resulting hybrid strains (referred to as globimar hybrids, from *globigii*-Marburg) as both recipients and donors to ascertain the relative importance of restriction and sequence nonhomology in limiting heterologous crosses for nonconserved markers. The crucial barriers to this rare mode of recombination are described in this paper. The accompanying paper (19) describes the mechanism of transformation of the heterologous sequences, known as intergenotes (41), by homologous and heterologous DNA.

MATERIALS AND METHODS

Strains. The bacterial strains used in these experiments are listed in Table 1. The parentage of markers in the globimar hybrids is indicated by the subscript s for *B. subtilis* and g for *B. globigii*.

Media. The broth medium used was Difco antibiotic medium no. 3 (Penassay broth; Difco Laboratories, Detroit, Mich.). For other purposes, M medium was Spizizen minimal salts (1) supplemented with 0.6% glucose and 25 μg of required amino acids per ml. Viable counts were performed on nutrient agar (Difco). For selective plating, M medium supplemented with glucose and selected amino acids was solidified with 1.5% agar.

Isotopes. [*methyl*-^{3}H]thymidine (40 Ci/mmol) was purchased from International Chemical and Nuclear Corp., Irvine, Calif.

Antibiotics and enzymes. Penicillin was purchased from E. R. Squibb and Son, Inc., New York, N.Y. Penicillinase was purchased as Neutrapen from Riker Laboratories, Northridge, Calif. Trimethoprim was a generous gift from Burroughs Wellcome Co., Research Triangle Park, N.C.

Mutant isolation. Cells growing in late log phase in Penassay broth were centrifuged, suspended in M medium with glucose, and UV irradiated to 1% survival (G. E. bulb, 3.7×10^3 ergs/cm^2 per s, maximal output 253.7 nm). Auxotrophic mutants were selected by the penicillin method (22) and detected by replica plating to selective media. Thymine-requiring mutants were obtained by mutagenesis with 20 μg of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Mann Research Laboratories, New York, N.Y.) per ml and selection in liquid culture with 200 μg of trimethoprim per ml, as described by Felkner (14).

DNA preparation. DNA was isolated by the method of Marmur (24) with the addition of ribonuclease before the second and subsequent deproteinizations. The concentration of the purified DNA solution was measured by the diphenylamine reaction (8).

Mechanical shearing of DNA. DNA was diluted to 40 μg/ml in 0.15 M NaCl–0.015 M sodium citrate (SSC) and hydrodynamically sheared by forceful passage 10 times through hypodermic needles of the following sizes: 18×, 23×, 25×, and 27×.

Sucrose gradient centrifugation. Sucrose gradient centrifugation was performed as described previously (18).

Competence induction and transformation.

TABLE 1. *Strains*[a]

Strain	Species	Genotype	Source
SB11	*B. subtilis*	*trpE thr-1*	Stanford[b]
SB19	*B. subtilis*	*str*^{r} prototroph	Stanford
SB32	*B. subtilis*	*hisB*	Stanford
SB202	*B. subtilis*	*aroB trpC hisB tyrA*	Stanford
SB863	*B. subtilis*	*aroB trpC tyrA hisA cys-1 leu*	Stanford
SB1023	*B. subtilis*	*aroB trpC hisB tyrA cys-1 leu*	Stanford
SB1070	*B. subtilis*	*thyA thyB*	Spontaneous *aroB*$^+$ revertant from SB749 (*aroB thyA thyB*)
SB1111	*B. subtilis*	*aroB trpC tyrA cys lys leu*	SB11 × SB1023, select *hisB*$^+$ low reversion for *trpC*
SB512	*B. globigii*	Prototroph	Stanford
SB1112	Hybrid	$(cys\ lys)_s\ (aroB^+ \rightarrow aroE^+)_g$	SB512 × SB1111
76-2	Hybrid	$(cys\ lys)_s\ trp_g$	UV irradiation of SB1112
76-3	Hybrid	$(cys\ lys)_s\ tyr_g$	UV irradiation of SB1112
135-1	Hybrid	$(cys\ lys\ thyA\ thyB)_s\ (aroB^+ \rightarrow aroE^+)_g$	NTG and TMP selection[c]
96-3-1	Hybrid	$(cys\ lys\ ser/gly)_s\ tyr_g$	UV irradiation of 76-3
99-12	Hybrid	$(cys\ lys\ leu\ hisA)_s\ trp_g$	76-2 × SB863
6-5	Hybrid	$(cys\ lys\ ser/gly)_s\ (hisB\ tyr)_g$	NTG mutagenesis and penicillin selection from 96-3-1
PheII	Hybrid	$(cys\ lys\ ser/gly)_s\ (tyrA\ aroE)_g$	NTG mutagenesis and penicillin selection from 96-3-1
38-1	Hybrid	$(cys\ lys\ ser/gly)_s\ (trp\ tyrA)_g$	99-12 × 6-5 selection *his*$^+$ trp_g

[a] For hybrid genotypes, the subscripts s or g, respectively, refer to the *B. subtilis* or *B. globigii* origin of the DNA sequence carrying the specific markers.

[b] Stanford, Stanford University Department of Genetics stock collection.

[c] NTG, Nitrosoguanidine; TMP, trimethoprim.

Competence induction and transformation were performed as described by Stewart (37).

Preparation of a crude extract from *B. globigii*. One liter of SB512 cells was grown to mid-log phase in Penassay broth, washed once with 100 ml of 0.1× SSC (0.15 M NaCl plus 0.015 M sodium citrate), and suspended in 5.5 ml of 6.6 mM tris(hydroxymethyl)-aminomethane (pH 7.4)–1 mM glutathione. All further steps were performed at 4°C. The cell suspension was sonically treated with a Branson sonifier, 3 A setting, for 10 min in 1-min segments. The sonically treated material was centrifuged at 30,000 rpm for 30 min, and the supernatant was withdrawn and stored at 4°C.

Assays of the *B. globigii* extract. (i) Viscosimetry. DNA (2.5 to 3.0 ml, diluted to 40 μg/ml in 6.6 mM tris(hydroxymethyl)aminomethane–6.6 mM $MgCl_2$–6.6 mM β-mercaptoethanol (pH 7.4), was added to an Ostwald viscosimeter and equilibrated at 30°C until readings were reproducible to within 0.1 s; 100 μl of extract was added, and readings were taken at frequent intervals. To terminate the reaction, 50 μg of deoxyribonuclease I per ml was added; terminal readings were obtained when no further change was measurable.

(ii) Bioassay. A 0.6-ml volume of DNA was equilibrated at 30°C as above; 5 μl of extract was added, and the mixture was incubated for 2 h. Biological activity was tested by transformation.

RESULTS

Transformation of *B. subtilis* recipients by *B. globigii* DNA. Initial attempts to transform *B. subtilis* competent cells with DNA from the *B. globigii* donor, SB512, for auxotrophic markers in nonconserved regions were, as expected, hampered by extremely low transformation frequencies. For several amino acid markers tested, the spontaneous reversion frequency was about 80% of the uncorrected "transformation frequency," with corrected values usually less than 5 transformants per ml at saturating DNA concentrations (4 to 8 μg/ml). There was no way of ensuring that a particular colony was a transformant rather than a revertant.

To solve this problem, it was decided to select for cotransformation of two linked markers simultaneously. The aromatic genetic region of the *B. subtilis* chromosome has been extensively mapped (27) and has been shown not to be conserved between *B. globigii* and *B. subtilis* (9). Accordingly, the *B. subtilis* strain SB1111 (*aroB trpC tyrA cys-1 lys*) was transformed at saturating concentrations by DNA from SB512 (*B. globigii*) or SB19 (*B. subtilis*), and double transformants for the linked aromatic markers, $aroB^+$ and $tropC^+$, were selected. Colonies were tested for cotransfer of the unselected linked marker, $tyrA^+$, by replica plating. The results of three experiments are summarized in Table 2. The E.T. of the heterologous cross is calculated as the ratio of heterologous to homologous transformation frequencies. *B. globigii* DNA can transform SB1111 to $aroB^+$-$trpC^+$ with an E.T. of 1.4×10^{-5} compared to homologous *B. subtilis* DNA. This value is typical of E.T. measurements made for other auxotrophic markers (*hisA, lys, tyrA*) where, however, the accuracy could not be guaranteed due to a high reversion frequency relative to transformation frequency.

TABLE 2. *Transformation of B. subtilis recipient cells by homologous and heterologous DNA*[a]

Donor	Recipient	Transformation frequency	E.T.	Cotransfer of $tyrA^+$ (%)
SB19	SB1111	3.1×10^{-3}		19
SB512	SB1111	4.3×10^{-8}	1.4×10^{-5}	96

[a] DNA from SB19 (*B. subtilis*) or SB512 (*B. globigii*) was used to transform SB1111 to $aroB^+$-$trpC^+$ as described in the text. Saturating DNA concentrations (8 μg/ml) were used. Transformation frequency is calculated as the ratio of transformants per milliliter to colony-forming units per milliliter. E.T. is the ratio of transformation frequencies using SB512 DNA relative to SB19 DNA.

The heterologous $aroB^+$-$trpC^+$ transformants have a very high frequency of cotransfer for $tyrA^+$ compared with homologous transformants (Table 2). This was unexpected, since it was thought that limited homology would increase the difficulty of incorporation of DNA as a function of length incorporated. Other workers, selecting markers in evolutionarily conserved regions of the chromosome, have found decreased cotransfer frequencies in heterologous crosses (5, 17, 31).

Although the *B. globigii* DNA is expressed in the heterologous transformants, it was not obvious that the transformation had occurred by integration into the *B. subtilis* chromosome and concurrent excision of the corresponding *B. subtilis* sequence; it was possible that the recipients were ectopically transformed strains, carrying both *B. globigii* and mutant *B. subtilis* aromatic region sequences at different positions on the chromosome or with the *B. globigii* sequences on an independent replicon. This was disproved by repulsion crosses. The *B. subtilis* parent, SB1111, is *trpC* $hisB^+$ *tyrA*. If this sequence remained in the new hybrid genome, transformation of a $trpC^+$ $hisB^+$ $tyrA^+$ recipient (SB32) to histidine prototrophy should yield some auxotrophs for tryptophan or tyrosine. Crude DNA was extracted from several hybrid colonies and used to transform SB32 to histidine prototrophy. Several hundred his^+ recombinants were replica plated to plates lacking tryptophan or tyrosine; no auxotrophs for these amino acids were obtained. In a control experiment, crude DNA from the parent, SB1111, was used in the same

cross; about 80% of his$^+$ transformants were cotransformed to tryptophan auxotrophy.

In the heterologous transformation, a lag period of 2 to 4 days passed before any transformants were visible as colonies. This lag period occurred for the heterologous transformation of all markers tested: *aroB-trypC*, *trypC*, *tyrA*, *hisA*, and *lys*. In contrast, transformant colonies were visible in 18 to 24 h when homologous SB19 DNA was used. When the hybrid recombinant colonies were picked and replated on fresh plates, they grew at normal rates, with colonies visible at 24 h. Thus, the lag period was observed only during the initial transformation with heterologous DNA.

Intergenote transformation: crosses involving globimar hybrids as recipients. To help distinguish whether enzymatic restriction or sequence nonhomology is the major barrier to these heterologous transformations, crosses were made using hybrids as recipients. One of the new hybrids, SB1112, carrying the entire aromatic region (*aroB*$^+ \rightarrow$ *aroE*$^+$) as a *B. globigii* intergenote, was mutagenized with UV irradiation, and, using penicillin selection, mutants in the intergenote requiring tryptophan (76-2) and tyrosine (76-3) were isolated. These new strains were made competent and transformed at saturating concentrations by SB19 and SB512 DNA. In each cross, two markers were selected: a suspected intergenote marker, trp_g or tyr_g, and a subtilis marker, lys_s. Transformation frequencies, E.T., and intergenote/*B. subtilis* marker transformation ratios were calculated (Table 3). The major result is that the *B. globigii* donor can transform the homologous intergenote marker with essentially normal frequency; for both trp_g and tyr_g, the E.T. was about 1.0. In contrast, heterologous transformation frequencies for the *B. subtilis* marker, lys_s, remained at the very low levels seen previously, with E.T. values around 10^{-6}. As seen by the trp_g^+/lys_s^+ and tyr_g^+/lys_s^+ ratios, then, the *B. globigii* donor's transforming efficiency is improved by a factor of almost 10^5 when the marker is located on a homologous intergenote in the predominantly heterologous recipient.

The lag period that had been observed in the *B. globigii* × *B. subtilis* crosses is not observed for *B. globigii* × hybrid crosses into the *B. globigii* intergenote; however, it remains in these crosses for the *B. subtilis* marker, lys_s. Thus, the lag is presumably caused by events during the initial interaction of donor DNA with the chromosome.

These results demonstrate the crucial role of sequence homology for efficient interspecies transformation. It is somewhat curious, then, that *B. subtilis* DNA continues to be able to transform the nonhomologous intergenote markers with high efficiency. The accompanying paper (19) gives evidence that this capability is retained due to the presence of homologous DNA sequences adjacent to the heterologous intergenote, which serve as recognition sequences for integration of the *B. subtilis* donor.

Enzymatic restriction does not act as barrier to *B. globigii* × *B. subtilis* transformation. The above results suggest that restriction does not play a role in limiting interspecies transformation. However, *B. subtilis* 168 strains do have a restriction system. Goodgal has reported the partial purification of a restriction endonuclease from *B. subtilis* 168 (Fed. Proc. **30**:1155, 1971), as have Wilson and Young (personal communication), though very high levels of exonuclease have hampered full purification. *B. subtilis* Marburg 168 strains have been shown to restrict ϕ105C grown on other bacillus species (34, 40); several other phages are not restricted. An attempt to demonstrate mutual restriction of ϕ105 and SPO2 growth between *B. subtilis* and

TABLE 3. *Transformation into heterozygote recipients*

Donor	Recipient	Marker	Transformation frequency	E.T.	Transformation ratios[a]	
					trp_g/lys_s	tyr_g/lys_s
SB512 (*B. globigii*)	76-2	trp_g	4.2×10^{-3}	1.1	7.5×10^4	
		lys_s	5.6×10^{-8}	3.2×10^{-6}		
SB19 (*B. subtilis*)	76-2	trp_g	3.8×10^{-3}		0.22	
		lys_s	1.7×10^{-2}			
SB512 (*B. globigii*)	76-3	tyr_g	8.0×10^{-3}	0.99		8.1×10^4
		lys_s	9.9×10^{-8}	3.3×10^{-6}		
SB19 (*B. subtilis*)	76-3	tyr_g	8.1×10^{-3}			0.27
		lys_s	3.0×10^{-2}			

[a] Transformation ratios were calculated from transformation frequencies using the same DNA to transform each of two markers.

B. globigii was frustrated by the finding that these phages do not absorb to *B. globigii* (data not shown).

Wilson and Young have purified two restriction endonucleases from *B. globigii*, *Bgl* I and *Bgl* II (personal communication). We prepared a crude extract from SB512 by sonic treatment of log phase cells and centrifugation to remove cellular debris. This extract degrades *B. subtilis* and calf thymus DNA, but does not attack *B. globigii* DNA, as tested by viscosimetric assay (Fig. 1). The absence of activity on SB512 DNA is not due to an inhibitory factor in that DNA preparation, since an equal mixture of SB512 and SB202 (*B. subtilis*) DNA was digested at approximately one half the rate of SB202 DNA alone (data not shown). Thus the crude extract shows *Bgl* I and -II enzymatic activity.

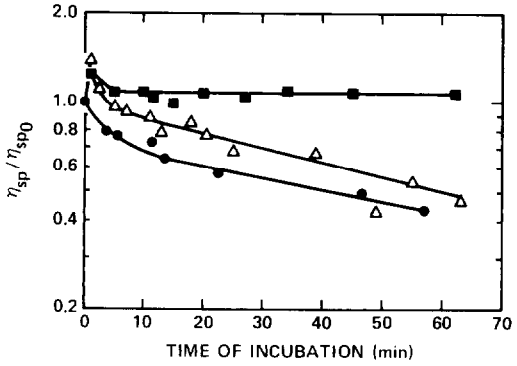

FIG. 1. *Effect of B. globigii crude extract on viscosity of DNA. DNA from SB512 (■), SB202 (△), and calf thymus (●) was prepared at 40 μg/ml in 6.6 mM tris(hydroxymethyl)aminomethane – $MgCl_2$ – β-mercaptoethanol (pH 7.4). The effect of a crude extract from SB512 on the viscosity of each solution was measured using Ostwald viscosimeters, as described in the text. The experimental measurement times, t, were divided by the terminal digestion time, t_D, after deoxyribonuclease digestion; η_{sp}, the specific viscosity, was calculated as $(t/t_D) - 1$. The percent decrease in specific viscosity from the undegraded sample viscosity, η_{sp_0}, was calculated as η_{sp}/η_{sp_0}. This ratio is plotted as a function of time of digestion.*

The effect of this extract on the biological activity of *B. subtilis*, globimar hybrid, and *B. globigii* DNA was measured in an attempt to demonstrate that the restriction-modification systems of *B. subtilis* and *B. globigii* recognize different sites. DNA was incubated with extract for 2 h at 37°C, then used to transform homologous and heterologous markers in *B. subtilis* and globimar hybrid recipients. The results are shown in Table 4. The extract did not alter the transforming activity of SB512 (*B. globigii*) DNA, either on a homologous intergenote marker or on *B. subtilis* recipients, although the very low transformation efficiencies in the latter case made this difficult to demonstrate conclusively (data not shown). The transforming activity of homogenotic *B. subtilis* DNA was reduced by the extract; for three homologous *B. subtilis* single markers, survival varied from 2 to 16%. Cotransfer of extended portions of the *B. subtilis* aromatic region from *aroB* to *tyrA* was far more sensitive to *B. globigii* restriction, whereas heterologous transformation of the tyr_g marker in the *B. globigii* intergenote in a hybrid recipient was almost completely inactivated. Most importantly, the *B. globigii* intergenote of the globimar hybrid was as sensitive to *B. globigii* extract as *B. subtilis* markers in the hybrid (such as $hisA^+$) or the *B. subtilis* homogenote. Thus, *B. globigii* sequences, including tyr^+, which are demonstrably resistant to the *B. globigii* restriction system when present in a homogeneous *B. globigii* donor, become sensitive to it after insertion as intergenote in a *B. subtilis* recipient and (presumably) *B. subtilis*-specific modification, showing that the restriction-modification sites of these species are indeed different. This was especially noticeable when the *B. globigii* intergenote transformed the heterologous aromatic region from *aroB* to *tyrA* in the *B. subtilis*

TABLE 4. *Effect of B. globigii extract on transforming activity of DNA*

Donor	Recipient	Marker	Transformation frequency Untreated	Transformation frequency Treated	Survival (%)
SB512 (*B. globigii*)	76-3	tyr_g	4.6×10^{-3}	5.4×10^{-3}	117
SB19 (*B. subtilis*)	SB202	$trpC_s$	2.5×10^{-4}	4×10^{-6}	1.6
	76-3	lys_s	1.9×10^{-2}	6.6×10^{-4}	3.5
	SB863	$hisA_s$	7.4×10^{-4}	1.2×10^{-4}	15.5
	SB202	$(aroB\text{-}tyrA)_s$	7.6×10^{-5}	5.3×10^{-8}	0.07
	76-3	tyr_g	5.3×10^{-3}	7.1×10^{-8}	0.001
76-2 (hybrid)	SB863	$hisA_s$	1.27×10^{-3}	1.44×10^{-4}	11.3
	76-3	tyr_g	9×10^{-3}	2.8×10^{-4}	3.1
	SB863	$(aroB\text{-}tyrA)_s$	4×10^{-5}	$<6 \times 10^{-9}$	<0.01

recipient, SB863; treatment with the *B. globigii* extract eliminated this transformation.

Assuming that the hybrid intergenote is modified to resistance to *B. subtilis* restriction, the relative efficiency of globimar hybrid and homogenote *B. globigii* DNAs in transforming intergenote markers in hybrid recipients can be calculated to assess the influence of *B. subtilis*-specific restriction as a factor limiting the biological activity of the foreign *B. globigii* DNA. To minimize differences in the physical state of the two DNAs and the effect of heterologous sequences adjacent to the intergenote in *B. globigii* × globimar hybrid crosses, both DNAs were sheared to varying degrees by forceful passage through hypodermic needles of different bore sizes. The weight-average molecular weight of each sheared sample was determined by neutral sucrose gradient centrifugation. Transformations were performed, selecting trp_g^+ in 99-12 and tyr_g^+ in 96-3-1, a *ser/gly* mutant derivative of hybrid 76-3 (Table 5). After shearing to an average size of 10^7 daltons or less, both DNAs were nearly equal in their ability to transform the homologous intergenote marker; with increasing size, the *B. globigii* donor became slightly less efficient than the globimar hybrid donor, reaching 50 to 70% efficiency at sizes around 10^8 daltons. Thus, the *B. globigii* donor transforms the homologous intergenote with high efficiency compared to the hybrid donor, especially when both DNAs are sheared to molecular weights smaller than the intergenote (see accompanying paper); restriction does not have an appreciable role in these heterologous transformations into the aromatic region of *B. subtilis*.

DISCUSSION

The very low transformation frequencies observed in *B. globigii* × *B. subtilis* crosses for auxotrophic markers are typical of those reported for nonconserved regions of the chromosome in other heterologous transformation systems (9). The initial lag period in appearance of transformed colonies is probably a physiological reflection of the difficulty of integration of highly heterologous DNA sequences, since the subsequently isolated hybrid colonies grow at normal rates and show no physiological defects. Alternatively, some secondary change in the recipient genome may be required before the donor gene products can function; however, UV irradiation of competent cells did not decrease the lag period or increase the frequency of heterologous transformants (data not shown).

We observed an increase in linkage of nonconserved aromatic markers in heterologous compared with homologous crosses (Table 2). Other workers studied heterologous transformation of linked antibiotic resistance markers in evolutionarily conserved regions of the chromosome; in contrast with our results, a significant reduction of linkage was observed (4, 5, 17, 31). This discrepancy could be explained by the higher degree of nonhomology between donor and recipient DNA in nonconserved as compared with conserved DNA sequences (9, 11). It is possible

TABLE 5. *Relative efficiency of transformation of B. globigii homogenote and globimar hybrid DNAs*

Donor	Recipient	Marker	Shear[a]	Weight-average molecular weight	Transformation frequency	Ratio SB512/135-1
135-1 (hybrid)	99-12 (hybrid)	trp_g	0	1.13×10^8	3.8×10^{-4}	
			18	4.6×10^7	2.0×10^{-4}	
			23	8.2×10^6	2.6×10^{-5}	
			27	3.6×10^6	1.5×10^{-5}	
SB512 (*B. globigii*)	99-12 (hybrid)	trp_g	0	8.1×10^7	2.8×10^{-4}	0.73
			18	4.3×10^7	1.3×10^{-4}	0.66
			23	1.1×10^7	3.4×10^{-5}	1.31
			27	5.9×10^6	1.4×10^{-5}	0.90
135-1 (hybrid)	96-3-1 (hybrid)	tyr_g	0	1.13×10^8	4.3×10^{-4}	
			18	4.6×10^7	1.9×10^{-4}	
			23	8.2×10^6	2.2×10^{-5}	
			27	3.6×10^6	1.8×10^{-5}	
SB512 (*B. globigii*)	96-3-1 (hybrid)	tyr_g	0	8.1×10^7	2.2×10^{-4}	0.50
			18	4.3×10^7	1.4×10^{-4}	0.77
			23	1.1×10^7	1.9×10^{-5}	0.86
			27	5.9×10^6	1.3×10^{-5}	0.73

[a] Size of hypodermic needle used for hydrodynamic shearing.

that in nonconserved regions of the chromosome, significantly longer pieces of donor DNA must undergo pairing with the recipient chromosome to effect successful integration than is necessary in conserved regions. In conserved regions, homology is fairly extensive as judged by hybridization experiments, and sequence mismatches could limit the extent of donor integration while still allowing integration of shorter homologous sequences around selected markers; this would permit fairly efficient transformation frequencies while reducing linkage (5). In nonconserved regions, stretches of sequence homology are quite rare, and heterologous integration may occur under different selection pressures than in conserved regions; here, the occasional homologous sequences would function to hold the much longer heterologous sequence in position until covalent joining of molecular ends and DNA replication or repair could render the sequence homogeneous. This would result in reduced transformation frequency but increased linkage, as has been observed. Chilton and McCarthy (9) did not see a significant reduction of linkage of markers in the poorly conserved region around *leu* during *B. globigii* × *B. subtilis* crosses.

When a *B. globigii* DNA sequence is present as an intergenote in a globimar hybrid recipient, the transforming activity of *B. globigii* DNA for the homologous intergenote marker rises by a factor of 10^5 to normal levels. At the same time, *B. globigii* transforming efficiency for markers located in *B. subtilis*-specific sequences of the hybrid remains very low, with a characteristic lag period before colonies arise; thus, the intergenote has not caused or resulted from a global change in the hybrid's ability to resist transformation by nonhomologous DNA. These data demonstrate the importance of sequence homology as the major barrier to heterologous transformation between these species. The slightly higher efficiency of globimar hybrid DNA over *B. globigii* DNA (Table 5) can be explained by the nonhomology of the *B. globigii* donor for *B. subtilis*-specific sequences adjacent to the intergenote. A similar finding was obtained by Biswas and Ravin (5), who found that pneumococcal DNA containing a streptococcal intergenote transformed a streptococcal recipient for markers carried by the intergenote with lower efficiency than homospecific streptococcal DNA; they referred to this as the "neighborhood effect." In our system, the *B. subtilis* neighborhood surrounding the *B. globigii* intergenote in the hybrid recipient interferes with integration of the homogenote *B. globigii* donor. The inverse situation explains the lower activity of *B. subtilis* DNA in transforming heterologous intergenote markers: as shown in the accompanying paper (19), the homologous neighborhood surrounding the intergenote allows integration of the heterologous DNA sequence at low efficiency.

The data in Tables 3 and 5 demonstrate that the *B. subtilis* enzymatic restriction system does not reduce the transforming activity of homogenote *B. globigii* DNA for aromatic region markers. There are several possible explanations for this lack of restriction. (i) The *B. subtilis* strains used in these studies may lack a restriction system. A restriction activity of *B. subtilis* 168 has been partially purified by Goodgal (Fed. Proc. **30**:1155, 1971), and restriction of phage growth by *B. subtilis* Marburg 168 strains has been reported for ϕ105C by two groups (34, 40); however, Trautner et al. (39) failed to show restriction of these or eight other phages using two different *B. subtilis* 168 strains. Due to technical difficulties, we were not able to determine whether our *B. subtilis* strains restrict ϕ105 or SPO2 grown on *B. globigii* hosts. The data of Fig. 1 and Table 4 show that the *B. globigii* restriction system does cleave *B. subtilis* and globimar hybrid DNA; hence, if our *B. subtilis* strains do carry a restriction-modification system, the sites recognized by the *B. globigii* and *B. subtilis* enzymes are not the same. (ii) The aromatic region of *B. globigii*, from *aroB* to *aroE*, may lack sites for the *B. subtilis* restriction endonuclease(s). In a previous paper (18) we showed that the restriction endonuclease *Eco*RI reduced the biological activity of *B. subtilis* DNA for all markers tested; the reduction was shown to depend on the size of DNA segment carrying the marker after cleavage and on the distance of the marker from the cleavage site. As discussed in the accompanying paper (19), the relationship between DNA size and transforming efficiency is nearly identical for *B. subtilis*, *B. globigii*, and globimar hybrid donors when each is tested on a homologous marker in a hybrid recipient; this would not be expected if the *B. globigii* donor were subjected to additional size reduction before integration due to a *B. subtilis* restriction activity. (iii) The restriction system is not active on transforming DNA. In our system *B. globigii* DNA transforms the homologous *B. globigii* intergenote in the predominantly *B. subtilis* recipient at normal frequencies, without any evidence of restriction. Trautner et al. (39) transformed *B. subtilis* 168 for $r^+ m_R^+$ phenotype from *B. subtilis* R. They found that 168 DNA ($r^- m_R^-$) and R DNA ($r^+ m_R^+$) transformed the 168 ($r^+ m_R^+$) recipient for auxotrophic markers with the same effi-

ciency. In contrast, phage infection, transduction, and transfection from an r^-m^- strain were severely restricted by the 168 ($r^+m_R^+$) recipient. In addition, transforming DNA from the 168 ($r^-m_R^-$) strain could be restricted in vitro by the R-specific restriction endonuclease, *Bsu*R, while DNA from the $r^+m_R^+$ strain was not affected by *Bsu*R in vitro. Gromkova and Goodgal (16) found several closely related species of *Haemophilus* that could transform each other with approximately equal efficiency, but from which restriction endonucleases with different specificities could be isolated. Furthermore, these enzymes were shown to cleave DNA from the other *Haemophilus* species in vitro. Similar results have been obtained in *Haemophilus* by Stuy (38).

Thus, it appears from results in three different systems that the restriction-modification system, although active in vitro on infecting, transducing, and transfecting DNA, may not be active on transforming DNA in vivo. In the *Bacillus* systems this may be related to the strandedness of the entering DNA inside the cell; transforming DNA is rendered single stranded during uptake (11), while infecting and transducing and transfecting DNA (3, 36) are present as double-stranded molecules after uptake. The ability of restriction endonucleases to cleave single-stranded DNA varies; *Bsu*R (7), *Eco*B (20, 23), *Hin*dII (35), *Hin*dV, *Hpa* II, *Alu* I, *Hae* II (6), and *Eco*RI (15) do not cleave single-stranded or denatured DNA, while *Hae* III and possibly *Hpa* II cleaved single-stranded DNA at the same sites as the corresponding double-stranded molecules (21). This single-stranded recognition capability, therefore, is not a general phenomenon, and it is not known whether such an activity occurs in vivo. Eisenstadt et al. (13) have partly characterized a protein from competent *B. subtilis* cells that binds to denatured DNA and protects it from digestion by single-strand-specific nucleases. This protein is not present in log phase cells, nor is it detectable in extracts of an asporogenous noncompetent mutant that was grown according to the normal competence regimen. This protein could function to protect single-stranded transforming DNA from restriction endonucleases as well as other nucleases.

ACKNOWLEDGMENTS

The expert technical assistance of P. Ruiz, J. Jennings, and P. Evans is gratefully acknowledged.

This study was supported by grants GM-00295 from the National Institute of General Medical Sciences, AI-5160 from the National Institute of Allergy and Infectious Diseases, CA-16896 from the National Cancer Institute, and NGR-05-020-004 from the National Aeronautics and Space Administration, and by an award from the Center for Interaction, Houston, Texas.

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